Different effect of cold storage and rewarming on three pH regulating transporters in isolated rat hepatocytes

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Forestal, Doris A., Judith Haimovici, and Pierre Haddad. Different effect of cold storage and rewarming on three pH regulating transporters in isolated rat hepatocytes. *Am. J. Physiol.* 272 (*Gastrointest. Liver Physiol.* 35): G638–G645, 1997.—Disturbances in hepatic pH homeostasis are thought to participate in the functional damage to liver grafts caused by the cold preservation and warm reperfusion necessitated by transplantation surgery. We have used an in vitro model of isolated rat hepatocytes suspended in cold University of Wisconsin (UW) solution and subsequently cultured at 37°C to evaluate liver cell pH regulatory mechanisms after cold preservation and rewarming. Cells were kept for up to 72 h in cold UW solution, and at 24-h intervals intracellular pH (pH_i) was measured after 60–90 min of warm culture by cytofluorometry using the fluorochrome 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein. When challenged with an alkaline load by isohydric HCO_3^--CO_2 steps, hepatocytes exhibited similar maximal pH_i values and recovered at the same rate, irrespective of cold storage time, indicating that Cl^-/HCO_3^- exchange activity is quite resistant to hypothermic storage and subsequent rewarming. In parallel studies, cells were subjected to an acid load by the NH_4Cl pulse technique in bicarbonate buffer containing 50 μM ethylisopropylamiloride to block Na^+/H^+ exchange. Despite similar nadir pH_i (lowest pH_i values due to acid load), the subsequent pH_i recovery rate that reflects Na^+-$(HCO_3^-)_n$ cotransport activity was increased significantly after hypothermic preservation. Hepatocytes were also perfused with a bicarbonate-free *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, and Na^+/H^+ exchange activity was evaluated using the same acid load protocol. Although cells always exhibited similar steady-state initial pH_i and nadir, the rate of pH_i recovery decreased significantly as a function of cold storage time in UW solution. Finally, intracellular buffering capacity was calculated from the sudden pH_i changes induced by HCO_3^--CO_2 steps or NH_4Cl pulses and was found to remain stable throughout the 72 h of cold preservation. Therefore, the results strongly suggest that cold preservation and rewarming disturb hepatocellular pH regulatory mechanisms by attenuating Na^+/H^+ exchange and increasing Na^+-$(HCO_3^-)_n$ cotransport, whereas Cl^-/HCO_3^- exchange is not affected.

University of Wisconsin solution; hydrogen ion concentration; ammonium chloride pulse; isohydric bicarbonate step; liver transplantation

SINCE THE ADVENT of preservation solutions such as University of Wisconsin (UW) solution, the success rate of liver transplants has significantly increased. This solution elaborated by Belzer and Southard (1) extended the safe cold preservation time of the donor liver from 8 h to ~20 h (13). Despite this improvement, a number of transplanted livers still suffer serious injury, independent of immune rejection (36), which results in borderline function or primary nonfunction (6, 13, 35). In these cases, graft injury is due mainly to damage that occurs in the liver during cold storage and/or during subsequent warm reperfusion (5). Several hypotheses have been brought forward to account for such "transplantation injury." Most studies have implicated the production of free radicals (9, 34), as well as disturbances of cellular calcium (22), volume (23, 33) and pH (11, 12, 19) homeostasis, as causes of this injury.

For instance, during cold preservation, the liver undergoes metabolic acidosis due to the anaerobic metabolism initiated during hypoxic hypothermia. An accumulation of lactic acid, protons, and other acid equivalents ensues (12). Such intracellular acidosis can activate lysosomal enzymes and may thus lead to lysosomal and, eventually, cellular lysis (8). Other studies have also shown that low cellular pH values can enhance the deleterious loss of membrane integrity induced by the production of oxygen free radicals during ischemia in liver tissue (19).

However, contradictory results have been obtained concerning the implication of pH changes in the damage caused to the liver by ischemia and reperfusion. Lie and Ukikusa (20) reported that an alkaline flush-out solution is more beneficial than physiological or acidic pH in prolonging the ischemic tolerance time of the liver. Others found no significant difference in liver cell viability within 48–72 h of cold storage when the extracellular pH of the *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered culture medium was varied between 5.0 and 8.5 (11). Recently, Gores et al. (15) showed that acidification of the UW storage solution improved the viability of perfused rat livers, whereas the viability of orthotopic rat liver transplants was worsened. Finally, two studies showed that hepatic intracellular pH (pH_i) becomes acid during anoxia (19) and simple cold storage (31) but returns rather rapidly to its normal value on reperfusion. Hence, it is still unclear how pH_i changes are involved in the deleterious effects of cold storage and warm reperfusion on liver grafts. Moreover, most studies on the effect of cold ischemia-reperfusion on liver pH homeostasis have been concerned with steady-state pH_i and not the activity of pH regulatory mechanisms.

Indeed, we know that steady-state pH_i results from an equilibrium between metabolic acid-base production and the activity of membrane transport proteins involved in pH_i homeostasis. In liver parenchymal cells, pH_i is maintained within narrow limits by the Na^+/H^+ exchanger (26, 28) and the Na^+-$(HCO_3^-)_n$ cotransporter (10, 14, 29), two proton-extruding proteins located at the basolateral membrane, and by the Cl^-/HCO_3^- exchanger (2, 24), an acid-loading transporter located at the apical membrane. The activities of the Na^+/H^+ and

Cl^-/HCO_3^- exchangers are modulated by pH_i (2, 28), and both participate in regulatory volume increase (RVI) mechanisms (7). Liver Na^+/H^+ exchange activity is also modulated by growth factors such as epidermal growth factor (27), but not by insulin (17). In contrast, little information is available regarding the regulation of Na^+-$(HCO_3^-)_n$ cotransport and Cl^-/HCO_3^- exchange by hormones (3).

Very few studies have directly addressed the impact of cold preservation and rewarming on hepatocellular pH regulatory mechanisms. We have therefore set out to study the effect of cold storage in UW solution on the three major membrane transporters implicated in hepatocyte pH homeostasis: the Na^+-$(HCO_3^-)_n$ symport and the Cl^-/HCO_3^- and Na^+/H^+ exchangers. Using an in vitro rat hepatocyte model, we found that the Cl^-/HCO_3^- exchanger activity remained rather stable, even after prolonged cold preservation in UW storage solution and subsequent short-term culture at 37°C. Na^+-$(HCO_3^-)_n$ cotransport activity was increased after hypothermic storage, indicating that this pH regulatory mechanism is disturbed by cold preservation and rewarming. In contrast, hepatocellular Na^+/H^+ exchange activity significantly decreased as a function of hypothermic storage time. Thus cold storage and rewarming specifically alter the Na^+/H^+ exchanger and the Na^+-$(HCO_3^-)_n$ symport, whereas the Cl^-/HCO_3^- exchanger appears to be more resistant. The observed perturbations in pH regulatory mechanisms may contribute to compromise the capacity of liver grafts to adequately face the important metabolic demands of the recipient and, hence, may participate in transplantation injury.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 150–175 g (Charles River Laboratories, St. Constant, PQ, Canada) were anesthetized with pentobarbital sodium (50 mg/kg ip; C. D. M. V., St. Hyacinthe, PQ, Canada) before surgery. All experimental protocols were accepted by the University Ethics Committee, and the animals were treated in accordance with the guidelines of the Canadian Council on the Care of Laboratory Animals.

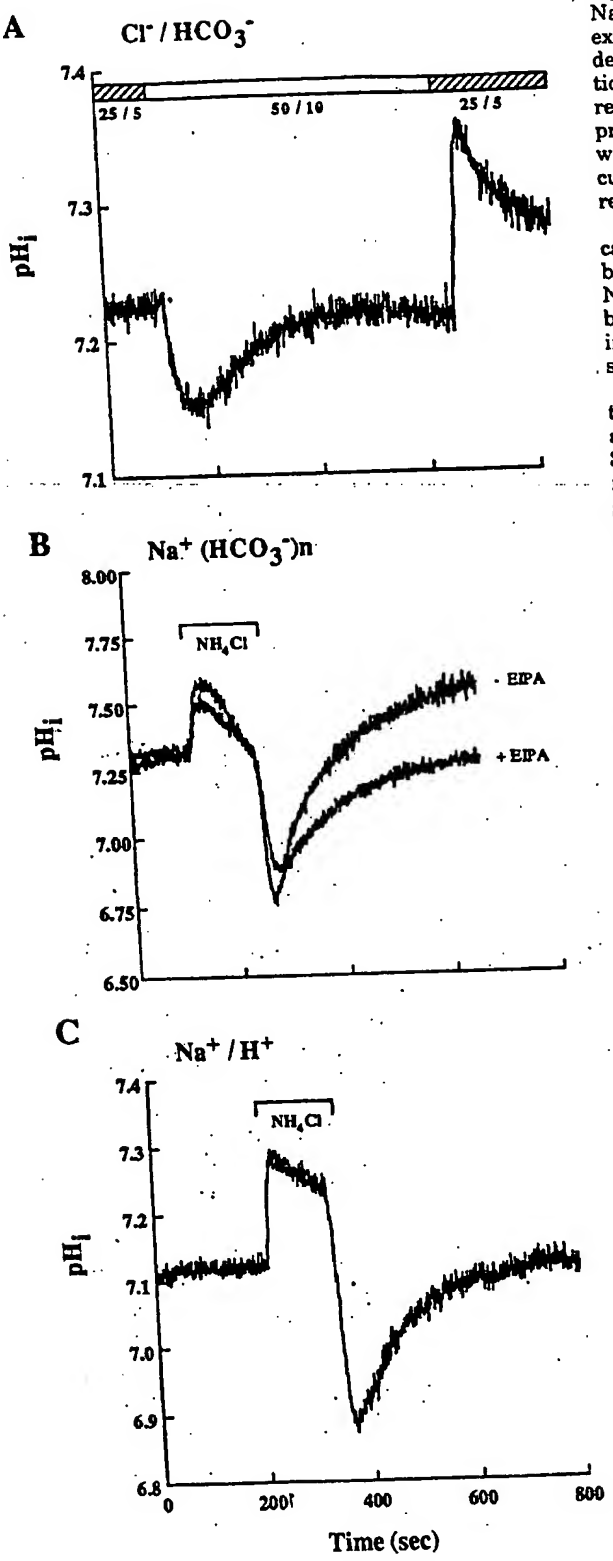
Hepatocyte preparation. Hepatocytes were isolated by collagenase perfusion of the liver, as described elsewhere (17). Purified hepatocytes were suspended in Belzer's UW cold storage solution (Dupont, Wilmington, DE) and allowed to stand undisturbed for 24, 48, or 72 h at 4°C in stoppered conical tubes containing ambient air. Control cells (0 h) were used immediately after isolation. After the designated time period, the hepatocytes were removed from the UW solution and resuspended in a culture medium devoid of hormones (Williams E culture medium, Sigma Chemical, St. Louis, MO) before cell viability was evaluated by exclusion of the dye trypan blue. Isolated hepatocytes were then seeded onto glass coverslips coated with rat tail collagen (type 4, Sigma Chemical) or Matrigel (Collaborative Biomedical Products, Bedford, MA) at a density of $8–10 \times 10^5$ cells/ml. The cells were incubated for 60–90 min at 37°C in a humidified 5% CO_2-95% O_2 atmosphere before they were used in the experiments. These conditions were selected to mimic in vitro the cold storage and warm reperfusion undergone by grafted livers in the clinic. This in vitro model has successfully been used by our laboratory (33) to study the impact of cold preservation and rewarming on hepatocyte volume regulatory function.

Measurement of pH_i. The pH_i was measured by ratio fluorescence microscopy using the proton fluorochrome 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF; Molecular Probes, Eugene, OR). Briefly, coverslips with adhered cells were placed on the stage of an inverted microscope (Olympus IMT-2) coupled to a spectrofluorometer (Deltascan RF-D4010, Photon Technology International, London, ON, Canada). In each experiment, groups of 6–10 closely apposed cells having well-defined cell borders and uniform cytoplasm were selected. After the measurement of autofluorescence, hepatocytes were covered for 5–10 min with a buffer containing the cell-permeant acetoxymethyl ester derivative of the fluorescent dye BCECF at 5 μM. Thereafter, cells were perifused (2 ml/min) with the appropriate thermostated and gassed solutions (see below). BCECF acetoxymethyl ester was prepared as a 1 mM stock solution in 95% ethanol. Ethylisopropylamiloride (EIPA; Research Biochemicals International, Natick, MA) was prepared as a 50 mM stock solution in dimethyl sulfoxide. Details of pH_i calibration and pH data acquisition and analysis are described elsewhere (17).

Measurement of Cl^-/HCO_3^- exchange activity. Cl^-/HCO_3^- exchange activity was measured by isohydric HCO_3^--CO_2 step, as previously described (2). Briefly, cells were first exposed for 20 min to a modified Krebs-Henseleit buffer (in mM: 95 NaCl, 5 KCl, 5 glucose, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 1 Na-pyruvate, 0.2 K_2HPO_4, 0.4 NaH_2PO_4) containing 50 mM $NaHCO_3$ gassed with 10% CO_2-90% O_2 (pH 7.4 at 37°C). On reaching a new steady-state pH_i, cells were alkalinized rapidly by simultaneous reduction of $NaHCO_3$ to 25 mM (isosmotically replaced by NaCl) and CO_2 to 5% (Fig. 1*A*), thus maintaining extracellular pH constant at 7.4. Previous studies have demonstrated that Cl^-/HCO_3^- exchange is the principal mechanism that returns pH_i toward baseline in such conditions (27). The initial recovery rate was obtained by measuring the tangent of the pH_i vs. time curve over the first 60 s after maximal pH_i was reached.

Measurement of Na^+-$(HCO_3^-)_n$ cotransport activity. Cells were perifused with a normal Krebs-Henseleit buffer (in mM: 120 NaCl, 5 KCl, 5 glucose, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 1 Na-pyruvate, 0.2 K_2HPO_4, 0.4 NaH_2PO_4) containing 25 mM $NaHCO_3$ and gassed with 5% CO_2-95% O_2 (pH 7.4 at 37°C). Na^+-$(HCO_3^-)_n$ cotransport activity was determined after an acid load induced by an NH_4Cl pulse, as described by Boron and De Weer (4). Briefly, cells were exposed for 2 min to a solution containing 20 mM NH_4Cl (isosmotic replacement of 20 mM NaCl). On removal of the NH_4Cl, pH_i is decreased and the recovery toward steady state is the result of the activity of acid-extruding mechanisms: Na^+-$(HCO_3^-)_n$ cotransport and Na^+/H^+ exchange. To isolate Na^+-$(HCO_3^-)_n$ cotransport activity from Na^+/H^+ exchange, NH_4Cl was washed from the cells with a normal Krebs-Henseleit solution containing the selective Na^+/H^+ exchange inhibitor EIPA at 50 μM (Fig. 1*B*) (37). Na^+-$(HCO_3^-)_n$ activity was determined from the initial pH recovery rate in the presence of EIPA obtained by measuring the tangent of the pH_i vs. time curve over the first 60 s after nadir pH_i (lowest point) was reached.

Measurement of Na^+/H^+ exchange activity. To minimize the effect of the Na^+-$(HCO_3^-)_n$ cotransporter and other HCO_3^--dependent mechanisms and, thus, to isolate the activity of the Na^+/H^+ exchanger, all experimental solutions used for this set of studies were devoid of HCO_3^- and contained 0.1 mM acetazolamide, an inhibitor of carbonic anhydrase, to limit endogenous HCO_3^- production. Thus hepatocytes were perifused with a buffer containing (in mM) 138 NaCl, 10 HEPES, 5 glucose, 3.8 KCl, 1.8 $CaCl_2$, 1.2 KH_2PO_4, 1.2



$MgSO_4$, and 1 Na-pyruvate (pH 7.4 adjusted at 37°C with NaOH) and gassed with 100% O_2. The activity of the Na^+/H^+ exchanger was also determined by the NH_4Cl pulse method described above (Fig. 1*C*). In such bicarbonate-free conditions, the rate of pH_i recovery subsequent to NH_4Cl removal reflects hepatocyte Na^+/H^+ exchange activity, as established previously by us and others (17, 28). The initial recovery rate was obtained by measuring the tangent of the pH_i vs. time curve over the first 60 s after nadir pH_i (lowest point) was reached (17).

Intracellular buffering capacity. The intrinsic buffering capacity (β_i) of the intracellular compartment was estimated by using the rapid pH_i changes observed on the withdrawal of NH_4Cl in bicarbonate-free conditions, as previously described by us (17) and others (30). Similarly, we estimated β_i from the instantaneous pH_i changes induced by isohydric HCO_3^--CO_2 steps, as described by Macri et al. (21).

Statistics. The effects of the various experimental conditions were evaluated by repeated measures analysis of variance (ANOVA) using the SigmaStat software package (Jandel Scientific, San Rafael, CA). At each preservation time, determinations were made in two to five different coverslips for a given cell preparation. We used the average of these multiple determinations to compare results, paired in time, for each cell preparation. Data are thus presented as means ± SE, with *n* representing the number of cell preparations (animals) rather than the number of experiments (coverslips studied). Because of the known dependence of Na^+/H^+ and Cl^-/HCO_3^- exchange activity on pH_i and of the variability in nadir or maximal pH_i values obtained after NH_4Cl pulses or isohydric HCO_3^-/CO_2 steps, we also performed an analysis of covariance between initial recovery rate (dpH_i/dt) and pH_i at the midpoint of the 1-min interval over which the recovery rate was measured. This analysis was performed using the SYSTAT software package (SYSTAT, Evanston, IL).

RESULTS

Hepatocyte viability. Cellular viability was evaluated at 24-h intervals, between 0 and 72 h of cold preservation in UW solution, by the trypan blue exclusion test. Hepatocyte viability decreased gradually and significantly from 87.4 ± 1.0% at 0 h to 82.7 ± 1.1, 78.8 ± 1.4,

Fig. 1. *A*: typical response of hepatocellular intracellular pH (pH_i) to isohydric HCO_3^--CO_2 steps. In cells subjected to an isohydric step from 25 mM HCO_3^--5% CO_2 (25/5) to 50 mM HCO_3^--10% CO_2 (50/10), pH_i decreased by ~0.1 pH unit, then gradually recovered. Cells were then alkali loaded by return to 25 mM HCO_3^--5% CO_2. This caused pH_i to rise by ~0.15 pH unit. Intracellular buffering capacity was estimated by this sudden change in pH_i, while Cl^-/HCO_3^- exchange activity was measured as initial slope of subsequent pH_i recovery over first 60 s. *B*: typical response of hepatocellular pH_i to an NH_4Cl pulse in Krebs-Henseleit buffer. Cells were subjected to 20 mM NH_4Cl in bicarbonate-containing Krebs-Henseleit buffer for 2 min. Acid load caused by withrawal of NH_4Cl is countered by coordinate action of Na^+/H^+ exchanger and Na^+-$(HCO_3^-)_n$ symport (−EIPA trace). Addition of 50 μM ethylisopropylamiloride (EIPA), a potent and selective inhibitor of Na^+/H^+ exchanger (37), allows measurement of component of recovery due to Na^+-$(HCO_3^-)_n$ cotransport activity (+EIPA trace). This activity was measured as initial slope of pH_i recovery in presence of EIPA over first 60 s. *C*: typical response of hepatocellular pH_i to an NH_4Cl pulse in HEPES buffer. Cells were subjected to 20 mM NH_4Cl in bicarbonate-free HEPES buffer containing 0.1 mM acetazolamide for 2 min. Sudden withdrawal of NH_4Cl caused pH_i to decrease abruptly by ~0.3 pH unit below initial pH_i. Intracellular buffering capacity was estimated by this rapid change in pH_i, while Na^+/H^+ exchange activity was measured as initial slope of subsequent pH_i recovery over first 60 s.

and 76.0 ± 1.5% at 24, 48, and 72 h, respectively ($P < 0.001$ for each preservation time by ANOVA), as also observed in a parallel study (33).

Hepatocellular Cl^-/HCO_3^- exchange. In a first series of experiments, we assessed the activity of the Cl^-/HCO_3^- exchanger after cold preservation and rewarming by submitting the hepatocytes to an acute alkaline load by isohydric HCO_3^--CO_2 steps (2). When exposed for 20 min to 50 mM HCO_3^--10% CO_2, steady-state pH_i equilibrated to 7.11 ± 0.01 in cells kept for 72 h in cold UW solution. This was lower than 7.16 ± 0.01 obtained in controls (0 h), but this effect did not reach statistical significance ("initial pH_i," $P = 0.130$; Table 1). When the perfusate HCO_3^--CO_2 content was suddenly decreased from 50 mM HCO_3^--10% CO_2 to 25 mM HCO_3^--5% CO_2, hepatocyte pH_i rapidly increased by ~0.15 pH unit (Fig. 1*A*). The extent of this alkalinization was not significantly affected by cold preservation time (P = NS by repeated measures ANOVA). Consequently, as with initial pH_i, the maximal pH_i reached after the isohydric step had a tendency to be lower as cold storage time increased ($P = 0.108$ for maximum pH_i; Table 1). However, initial pH_i recovery rates from this alkaline maximum remained rather constant, irrespective of hypothermic preservation time (P = NS; Table 1). Similarly, steady-state pH_i values reached 10–15 min later were equivalent in all groups ("final pH_i," $P = 0.493$; Table 1). Because Cl^-/HCO_3^- exchange activity is modulated by pH_i (2), we also performed an analysis of covariance between the initial rate of pH_i recovery and pH_i (at the midpoint of the 1-min measurement interval) as a function of hypothermic preservation time. This statistical analysis confirmed that the activity of the Cl^-/HCO_3^- exchanger was not significantly affected by storage in cold UW solution for up to 72 h ($P = 0.791$; Table 1).

Hepatocellular Na^+-$(HCO_3^-)_n$ cotransport. In a second series of experiments, we determined the activity of the Na^+-$(HCO_3^-)_n$ symport in cells maintained for up to 72 h in cold UW solution and rewarmed by short-term culture at 37°C. Hepatocytes perifused with a normal Krebs-Henseleit buffer generally exhibited steady-state pH_i values between 7.2 and 7.3. This baseline pH_i decreased significantly as cold preservation time exceeded 24 h ($P = 0.027$ by repeated measures ANOVA; Table 2). On administration of 20 mM NH_4Cl, pH_i rapidly increased due to the entry of NH_3 and its protonation to NH_4^+ (Fig. 1*B*). Return to normal buffer caused pH_i to drop by 0.3–0.4 pH unit, irrespective of the period of hypothermic storage (Table 2). The subsequent recovery rate is known to reflect Na^+-$(HCO_3^-)_n$ cotransport activity when Na^+/H^+ exchange inhibitors are present (14). In the presence of 50 μM EIPA, the initial rate of pH_i recovery increased significantly when cells were hypothermically preserved in UW solution ($P = 0.002$ by repeated measures ANOVA; Table 2) with the exception of the 48-h time point. Final pH_i values measured 10 min after the withdrawal of NH_4Cl had a tendency to decrease as a function of preservation time, but this effect did not reach statistical significance ($P = 0.182$ by repeated measures ANOVA; Table 2).

Hepatocellular Na^+/H^+ exchange. In the last series of experiments, we evaluated the activity of the Na^+/H^+ exchanger as a function of cold storage time. Steady-state pH_i of cells kept for up to 72 h in UW solution at 4°C and subsequently incubated at 37°C was stable. Indeed, hepatocytes perfused with bicarbonate-free HEPES buffer (see MATERIALS AND METHODS) exhibited baseline pH_i of ~7.1, irrespective of cold preservation time ($P = 0.803$; Table 3).

When cells were challenged with 20 mM NH_4Cl for 2 min, pH_i decreased by ~0.3 pH unit (Fig. 1*C*). This nadir pH_i (lowest point) was similar in control cells and in hepatocytes stored for 24–72 h in cold UW solution ($P = 0.978$; Table 3). On the other hand, final pH_i values reached ~10 min after the removal of NH_4Cl had a tendency to decrease in cells stored in the cold for ≥48 h, but this effect did not reach statistical significance ($P = 0.115$; Table 3).

As illustrated in Fig. 2, the initial rate of recovery from nadir pH_i similarly decreased as a function of cold preservation time from 0.100 ± 0.010 to 0.071 ± 0.010 pH unit/min between 0 and 72 h (Table 3). When evaluated by repeated measures ANOVA, this effect did not reach statistical significance because of the large

Table 1. *Alkaline load by isohydric HCO_3^--CO_2 step*

Time in UW Solution, h	pH_i Initial	pH_i Maximum	pH_i Final	Initial Recovery Rate, pH unit/min	n
0	7.16 ± 0.01	7.30 ± 0.02	7.18 ± 0.01	−0.048 ± 0.010	11
24	7.14 ± 0.02	7.28 ± 0.02	7.18 ± 0.02	−0.039 ± 0.005	11
48	7.13 ± 0.02	7.29 ± 0.02	7.18 ± 0.02	−0.046 ± 0.010	11
72	7.11 ± 0.01	7.24 ± 0.02	7.15 ± 0.01	−0.041 ± 0.010	10
P (ANOVA)	0.130	0.108	0.493	0.594	
P (ANCOVA)				0.791	

Values are means ± SE; n, no. of cell preparations (animals), rather than no. of experiments. Initial intracellular pH (pH_i) recovery rate after alkaline load was measured by calculating slope over 1st min. Statistical significance between group means was analyzed by one-way repeated measures analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was also performed on initial recovery rate as a function of preservation time using pH_i (midpoint of measurement interval) as covariable, since Cl/HCO_3^- exchange activity is known to be modulated by pH_i (2). UW, University of Wisconsin.

Table 2. *Acid load by NH_4Cl pulse in Krebs-Henseleit buffer*

Time in UW Solution, h	pH_i Initial	pH_i Nadir	pH_i Final	Initial Recovery Rate, pH unit/min	n
0	7.23 ± 0.02	6.91 ± 0.04	7.16 ± 0.03	0.098 ± 0.010	7
24	7.20 ± 0.03	6.81 ± 0.06	7.16 ± 0.05	0.159 ± 0.019*	7
48	7.17 ± 0.02*	6.83 ± 0.04	7.12 ± 0.03	0.114 ± 0.011	7
72	7.17 ± 0.02*	6.76 ± 0.06	7.10 ± 0.04	0.182 ± 0.003*	7
P (ANOVA)	0.027	0.156	0.182	0.002	

Values are means ± SE; n, no. of cell preparations (animals), rather than no. of experiments. Initial pH_i recovery rate after exposure to a 2-min 20 mM NH_4Cl pulse was measured in presence of 50 μM ethylisopropylamiloride. Statistical significance between group means was analyzed by one-way repeated measures ANOVA. * Significantly different from unstored controls (0 h), $P < 0.05$ (Bonferroni's method).

Table 3. *Acid load by NH_4Cl pulse in HEPES buffer*

Time in UW Solution, h	pH_i Initial	pH_i Nadir	pH_i Final	Initial Recovery Rate, pH unit/min	n
0	7.10 ± 0.02	6.84 ± 0.03	7.06 ± 0.02	0.100 ± 0.010	17
24	7.10 ± 0.01	6.84 ± 0.03	7.06 ± 0.01	0.079 ± 0.010	17
48	7.09 ± 0.01	6.82 ± 0.03	7.04 ± 0.02	0.079 ± 0.010	16
72	7.09 ± 0.02	6.83 ± 0.03	7.02 ± 0.02	0.071 ± 0.010	16
P (ANOVA)	0.803	0.978	0.115	0.313	
P (ANCOVA)				0.049	

Values are means ± SE; *n*, no. of cell preparations (animals), rather than no. of experiments. Initial pH_i recovery rate was measured after exposure to a 2-min 20 mM NH_4Cl pulse. Statistical significance between group means was analyzed by one-way repeated measures ANOVA. ANCOVA was also performed on initial recovery rate as a function of preservation time using pH_i (midpoint of measurement interval) as covariable, since Na^+/H^+ exchange activity is known to be modulated by pH_i (28).

variability of the data. However, part of this variability could be related to the known dependence of the Na^+/H^+ exchanger activity on pH_i (28), as reflected in our conditions by the nadir pH_i reached after the NH_4Cl pulse in individual experiments. We therefore performed an analysis of covariance between initial pH_i recovery rate and pH_i (at the midpoint of the 1-min measurement interval) as a function of hypothermic preservation time. Figure 3 presents the relationship between initial pH_i recovery rate and pH_i for controls (0 h, Fig. 3*A*) and for cells kept for 24–72 h in cold UW solution (Fig. 3, *B–D*). Analysis of covariance confirmed ($P < 0.001$) that dpH_i/dt was inversely related to pH_i, as found in several cells (25) including those of the liver (28). It further demonstrated that the rate of pH_i recovery decreased significantly as a function of cold preservation time ($P = 0.049$).

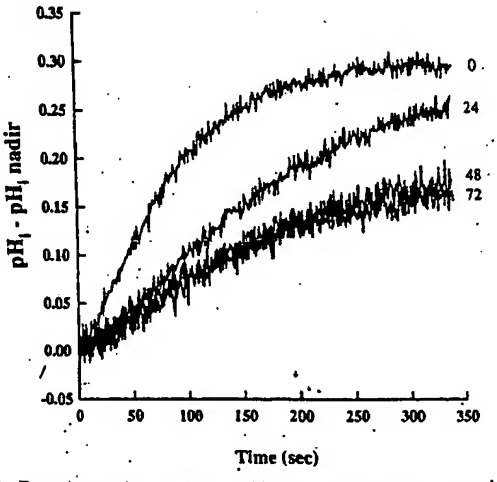


Fig. 2. Representative recovery of hepatocellular pH_i from an acid load in HEPES buffer as a function of cold storage time. Representative pH_i changes obtained on withdrawal of NH_4Cl in control unstored cells (0 h) are compared with changes observed in cells maintained for 24, 48, and 72 h in cold UW solution before culture at 37°C. For sake of clarity, traces were normalized to their respective nadir pH_i (pH_i − nadir pH_i) and were chosen for their slopes that reflected overall mean values obtained in each group.

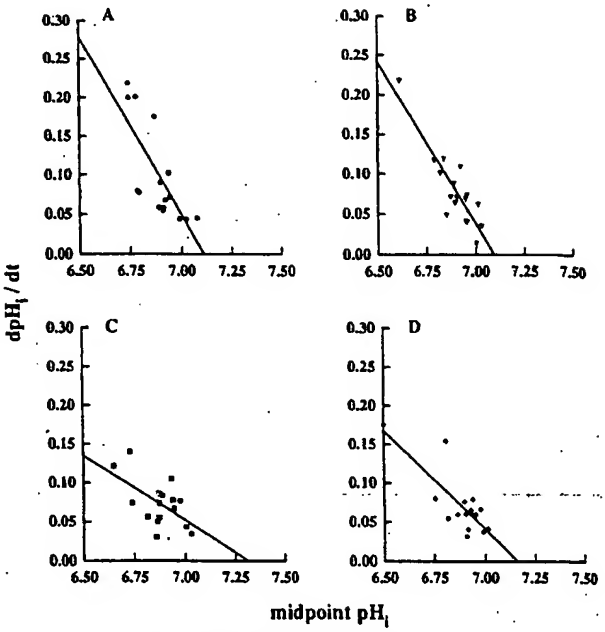


Fig. 3. Relationship between Na^+/H^+ exchange activity and pH_i as a function of cold preservation time in UW solution. Initial pH_i recovery rates (dpH_i/dt) observed for each cell preparation are plotted against midpoint of pH_i interval corresponding to 1-min measurement interval. Results of unstored control hepatocytes (*A*) are compared with those obtained in cells maintained for 24 h (*B*), 48 h (*C*), and 72 h (*D*) in cold University of Wisconsin (UW) solution before culture at 37°C. Analysis of covariance for repeated measures was carried out on daily averages of each preservation time in each cell preparation. When known influence of pH_i on dpH_i/dt (2, 28) is taken into account and confirmed by such statistical analysis ($P < 0.001$), preservation time significantly decreases Na^+/H^+ exchange activity ($P = 0.049$). As preservation time increases, relationship between dpH_i/dt and pH_i (slope of curves) also appears to be blunted, but this effect does not reach statistical significance ($P = 0.165$).

Intrinsic buffering capacity. It is possible to utilize the instantaneous changes in pH_i induced by the sudden addition or withdrawal of weak acids/bases, such as NH_4Cl or $NaHCO_3$ used in our experiments, to approximate the β_i of living cells (21, 30). We have calculated β_i using the pH_i changes induced by the sudden withdrawal of NH_4Cl and by the isohydric step from 50 mM HCO_3^--10% CO_2 to 25 mM HCO_3^--5% CO_2. As described in Table 4, β_i was unaffected by cold preservation in UW solution for up to 72 h, irrespective of the parameter used to approximate its value. However, as expected (2), β_i was greater at more acidic pH_i values (NH_4Cl pulse) than at more basic ones (HCO_3^--CO_2 step).

DISCUSSION

The results obtained during this study confirmed that the UW preservation solution effectively preserves gross hepatocellular viability for extended periods of time. As observed in a parallel study (33), the gross viability of hepatocyte suspensions decreases only by ~10%, even after 72 h in cold UW solution, compared with unstored controls (0 h). However, despite the

Table 4. *Intracellular buffering capacity*

Time, h	NH_4Cl Pulse			HCO_3^--CO_2 Step		
	β_i	Midpoint pH_i	n	β_i	Midpoint pH_i	n
0	106.7 ± 17.0	7.03 ± 0.02	17	54.9 ± 6.2	7.23 ± 0.01	11
24	101.1 ± 15.8	7.03 ± 0.02	17	49.9 ± 5.1	7.21 ± 0.02	11
48	89.9 ± 13.9	7.04 ± 0.02	16	48.8 ± 6.2	7.19 ± 0.02	11
72	91.1 ± 16.0	7.04 ± 0.02	16	48.7 ± 4.7	7.17 ± 0.01	10
P (ANOVA)	0.162	0.940		0.430	0.236	

Values are means ± SE; n, no. of cell preparations (animals), rather than no. of experiments. Intrinsic buffering capacity (β_i) was calculated using sudden pH_i changes induced by NH_4Cl removal (Fig. 1*C*) or by return to 25 mM HCO_3^--5% CO_2 after isohydric steps (Fig. 1*A*) and is expressed in mM/pH unit. In both cases, midpoint pH_i represents middle of interval over which sudden pH_i changes were observed. Statistical significances between group means were analyzed by one-way repeated measures ANOVA.

maintenance of an apparently good cell viability during cold preservation, a significant proportion of liver grafts display inappropriate function after transplantation (6, 13, 35). In a recent review of clinical studies, several risk factors were associated with poor prognosis after liver transplantation (36). Among these, the time spent by the donor organ in cold UW solution was considered important in relative and absolute terms (36). Thus studies aimed at better understanding the disturbances that underlie the dysfunction of liver transplants should provide avenues for the improvement of preservation solutions and, hence, of liver transplantation.

Among the several hypotheses advanced to explain such liver dysfunction, perturbations of hepatocellular pH homeostasis have been the focus of the present studies. To that effect, we have used an in vitro model based on purified isolated rat hepatocytes preserved in cold UW solution and subsequently cultured in warm medium to mimic the cold preservation and warm reperfusion undergone by transplanted organs. Several studies by other laboratories have also used isolated cells to gain insight into liver cell function after cold preservation in UW solution (22, 32, 38), including studies on the impact of external pH on such function (11). However, these studies and others (19, 31) were concerned only with steady-state pH changes and not with pH regulatory behavior.

The present studies were therefore concerned with the impact of in vitro cold preservation and rewarming on the three major transporters involved in liver pH_i regulation: the Na^+-$(HCO_3^-)_n$ cotransporter and the Cl^-/HCO_3^- and Na^+/H^+ exchangers. Our results clearly demonstrate that preservation of suspended hepatocytes for up to 72 h in cold UW solution followed by short-term culture at 37°C does not significantly modify the capacity of liver parenchymal cells to counter a sudden alkaline load. It was previously shown that recovery from such alkaline loads was Na^+ independent, electroneutral, and strictly Cl^- dependent, implying that Cl^-/HCO_3^- exchange is the most important membrane transporter involved in such acid-loading compensatory ion movements (2). Our results now indicate that this transporter is quite resistant to prolonged hypothermia and subsequent rewarming.

In contrast, the rate of pH_i recovery from similar acidic nadir values induced by the classical NH_4Cl pulse technique (4) in HEPES buffer was 20–30% more sluggish in cells preserved for 24–72 h in UW solution than in unstored controls. Because Na^+/H^+ exchange is the only pH regulatory mechanism active in such experimental conditions [as established previously with EIPA (17)], this result strongly suggests that the activity of the hepatocellular Na^+/H^+ exchanger is gradually and significantly decreased as a function of the time spent by the cells in cold UW solution. This was confirmed to be statistically significant by analysis of covariance that took into consideration the variations in dpH_i/dt that were related to variations in nadir pH_i. Indeed, it is well known that the activity of the Na^+/H^+ exchanger in liver (28) and other cells (25) increases as pH_i decreases. Moreover, our experiments clearly show that the β_i of rat hepatocytes, measured in two pH_i ranges, is not significantly modified by cold storage in UW solution for up to 72 h. Our results thus imply that, at any given pH_i, the acid-extruding activity of the Na^+/H^+ exchanger will be decreased as cold preservation time is increased. This is also supported by the tendency ($P = 0.115$) of pH_i to recover to gradually lower steady-state values after NH_4Cl pulses (final pH_i in Table 3) as cold storage time increases.

Our results are in contradiction with the preliminary studies reported by Hebling et al. (18). Unlike the present studies, they used the EIPA-sensitive component of pH_i recovery from an acid load induced by a 20 mM NH_4Cl pulse in a bicarbonate-containing buffer to evaluate hepatocellular Na^+/H^+ exchange activity. They found no difference in initial recovery rates when the rat cells were stored for up to 48 h in UW solution and subsequently incubated at 37°C, thus suggesting that the Na^+/H^+ exchange activity was well preserved. However, in these studies, unlike those presented here, fetal calf serum was added to the culture medium during rewarming. Such fetal bovine serum contains several growth factors (which are present in higher concentrations than in the adult) and is one of the most powerful stimulators of the Na^+/H^+ exchanger (25). It is therefore possible that Hebling et al. could not observe the reduced activity presented here because the exchanger was maximally stimulated by serum in their incubation medium. This raises the interesting possibility that, despite its reduced activity as a function of cold storage time, Na^+/H^+ exchange activity maintains its full capacity to be modulated by mitogens. It must also be stressed that our experiments were carried out on cells chosen by morphological criteria for their closest correspondence to normal freshly isolated hepatocytes (see MATERIALS AND METHODS). Thus it is probable that we are underestimating the true severity of the damage to the Na^+/H^+ exchanger caused by cold preservation and rewarming.

Moreover, using identical experimental conditions, our laboratory recently found that the rate of RVI observed after osmotically induced hepatocyte shrink-

age decreases significantly as a function of cold storage time (33). Such RVI in liver cells proceeds by simultaneous increases in Na^+/H^+ and Cl^-/HCO_3^- exchange activity accompanied by an increase in Na^+-K^+-ATPase activity and a reduction in K^+ permeability (16). The reduction in Na^+/H^+ exchange activity observed in the present studies can thus play a significant role in this phenomenon and may participate in liver dysfunction after transplantation.

The molecular mechanisms underlying the compromised activity of the Na^+/H^+ exchanger after cold preservation and rewarming remain unknown. One possibility is that the reduction in Na^+/H^+ exchange activity as a function of cold storage time stems from a decreased allosteric regulation by intracellular protons. Alternatively, a decreased maximal transport rate may occur through a reduction in the number of Na^+/H^+ exchangers in the membrane or through a decrease in the intrinsic activity of each transport molecule. Further experiments are required to fully address this point.

We have also evaluated the activity of the Na^+-$(HCO_3^-)_n$ cotransporter by measuring the recovery from an acid load in bicarbonate buffer containing the Na^+/H^+ exchange inhibitor EIPA (37). Our laboratory has confirmed that EIPA, at the concentration used here, completely abolishes hepatocellular Na^+/H^+ exchange (17). Our results thus demonstrate that Na^+-$(HCO_3^-)_n$ transport activity increases substantially and significantly after 24 and 72 h of cold storage in UW solution, whereas it is closer to control values after 48 h of hypothermic preservation. This indicates that the Na^+-$(HCO_3^-)_n$ cotransporter is also perturbed by cold preservation and rewarming. As mentioned above, the hepatocytes selected for study were probably the best preserved at all storage times. It is possible that the increased activity of the Na^+-$(HCO_3^-)_n$ cotransporter observed here may have helped counter the failing Na^+/H^+ exchange activity and given these cells a survival advantage.

Despite the maintenance of appropriate Cl^-/HCO_3^- exchange, the altered function of the Na^+-$(HCO_3^-)_n$ symport and the gradual decrease in Na^+/H^+ exchange activity suggest that hepatocellular pH regulatory mechanisms may not function optimally to maintain pH_i within its narrow physiological range as cold storage time increases. This contention is supported by the observation that baseline pH_i values in bicarbonate buffer (initial pH_i in Table 2) were significantly lower in cells preserved for >24 h. This is also supported by the tendency ($P = 0.130$) of pH_i to return to lower steady-state values after the acid load induced by the isohydric step from 25 mM HCO_3^--5% CO_2 to 50 mM HCO_3^--10% CO_2 (initial pH_i in Table 1, Fig. 1*A*) as cold storage time increases. Indeed, this recovery depends on the coordinate activity of the Na^+/H^+ exchanger and of the Na^+-$(HCO_3^-)_n$ symport (14). One can thus postulate that such slight but prolonged intracellular acidosis can result from active hepatic metabolism and secretion after cold preservation and rewarming. This could have deleterious effects on liver function and may participate, at least in part, in the initial poor function or dysfunction observed in a significant proportion of liver grafts after transplantation (6, 13, 35, 36).

Finally, clinical studies have found that preservation times >30 h in cold UW solution represent an absolute risk factor for poor transplantation prognosis (36). This corresponds to the time when altered pH (present studies) and volume (33) homeostasis become most evident in our in vitro model. Evidently, further studies with perfused livers and animal transplantation models are required to ascertain the significance of our findings for liver transplantation. It is nonetheless justifiable and worthwhile to pursue in vitro studies, because such models may represent a useful, cost-effective, and rapid method to deepen our understanding of the mechanisms underlying liver dysfunction after cold preservation and rewarming.

Once a parallel can be established with organ and in vivo models, in vitro models could also serve to screen modifications of preservation or rewarming conditions susceptible to improve the success rate of transplantation.

In conclusion, our studies suggest that hypothermic preservation of liver cells followed by rewarming attenuates their Na^+/H^+ exchange activity and disturbs their Na^+-$(HCO_3^-)_n$ cotransport activity but does not affect their Cl^-/HCO_3^- membrane transport function. This perturbation of pH regulatory mechanisms may participate in rendering certain transplanted organs (especially those preserved for long periods in cold UW solution) more prone to pH_i disturbances stemming from active metabolism after reperfusion.

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